Influence of mulberry nutrition on the regulation of haemolymph cation levels in the silkworm, *Bombyx mori*

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Abstract: Changes in the levels of haemolymph cations of 5th instar larvae of Pure Mysore, NB_4D_2 and CSR_2 fed on M-5, S-36 and V-1 mulberry varieties were investigated. The larval haemolymph showed low Na^+ and very high K^+ and Mg^{2+} concentrations and moderately high Ca^{2+} like the mulberry leaf. The haemolymph cation levels increased significantly during the active feeding period of the larva. A negative correlation existed between the levels of cations in haemolymph and excreta. The haemolymph cation levels were held by controlled excretion of the cations. The rates of elimination of cations were lower during feeding stages than during non feeding stages. The haemolymph cation levels of bivoltine races were about 30% higher than the multivoltine PM. The impact of race was significantly more than that of mulberry variety or ontogeny in upward changes in haemolymph cations.
Key words: *Bombyx mori*; mulberry leaf; nutrition; haemolymph; cation level

1 INTRODUCTION

Maintenance of appropriate fluid and electrolyte homeostasis in insects is essential for their normal development, growth and reproduction (Pannabecker *et al.*, 1992). Potassium, magnesium, calcium and sodium are the major cations present in the order of decreasing abundance in insect hemolymph. Diet and environment greatly influence cationic composition of the haemolymph. Food is the only source of ions in phytophagous insects which cannot consume or imbibe fluids directly. Haemolymph is a dynamic fluid tissue with close metabolic relationship with other tissues and organs. Terrestrial insects are confronted with the problem of maintaining fluid and ionic composition of haemolymph plasma at levels best suited for the various physiological activities at different stages in their life cycle. The silkworm in the 5th instar is fed with medium coarse mulberry leaf which contained relatively less water and electrolytes than the leaves offered to young age silkworms. The ionic composition of the insect haemolymph is patterned after the mineral composition of the leaf of the host plant (Florkin and Jeuniax, 1974). Calcium, iron, magnesium, manganese, phosphorus, potassium and zinc are required for normal growth and development of the mulberry silkworm (Ito, 1967). Minerals account for 10% of dried mulberry leaves but 28% of the silkworm (Ito, 1978). Absorption of nutrients depends on the composition of the diet and midgut enzyme activity and permeability (Primor and Zlotkin, 1980; Harvey, 1982). K^+ and Mg^{2+} levels tend to be higher in the haemolymph of leaf-eating caterpillars of lepidopterous insects reflecting the levels of the elements in plant tissues (Chapman, 1998). The ionic composition of haemolymph is maintained by balancing absorption of minerals through the midgut epithelium and excretion through Malpighian tubule. The influence of ontogeny, silkworm race and mulberry variety on changing cation levels of haemolymph was examined in the context of physiological or economic significance.

2 MATERIALS AND METHODS

2.1 Mulberry varieties

Three mulberry genotypes, *viz.* M-5, S-36 and V-1, were planted in the mulberry garden of the Department of Sericulture, University of Mysore, Mansagangotri, Mysore during 1998. The plots measured 5 m × 4 m with a planting space of 0.9 m × 0.9 m. The pH of the red sandy loamy soil used for plantation is 6.9. The experiment was laid out in randomized complete block design with three replications. Recommended doses of farm yard manure of 20 MT/ha/yr for S-36 and M-5 plantation and 40 MT for V-1 plantation in two split doses, and chemical fertilizers at the rate of 300 kg: 120 kg: 120

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kg of NPK for S-36 and M-5 plantation and 350 kg: 140 kg: 140 kg of NPK for V-1 plantation were applied. The chemical fertilizers were applied in five equal split doses of nitrogen, and two each of phosphorus and potassium applied to 1st and 3rd crops. Around 1 136.25 m^{-3} of water was provided per acre at intervals of 10 d in an approximately 70 d crop period for the plots of all the three mulberry varieties.

2.2 Silkworm rearing

Ten disease free layings of PM, NB_4D_2 and CSR_2 were brushed and reared separately as per the standard rearing technique of Dandin *et al*. (2003) on fresh leaves of the mulberry varieties of V-1, S-36 and M-5. The experiment was conducted in summer season (March-April) when the ambient temperature was 30 – 35℃. Na^+, K^+, Ca^{2+} and Mg^{2+} levels in the haemolymph of the silkworms, fed on the leaves of different mulberry varieties, were estimated by flame photometry.

2.3 Haemolymph collection

Haemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea by cutting the first proleg of larva, from the larvae in 4th moult, post moult, and feeding and spinning larvae and centrifuged at 3 000 g for 10 min at 4℃. The supernatants were used in the cation estimations.

2.4 Estimation of cations

To 1 mL of haemolymph, 10 mL of diacid (nitric acid∶perchloric acid = 9∶4) was added. Similarly, known quantity of dried mulberry leaf powder and excreta were digested using digestion chamber (Digester 1009) until a clear solution was obtained. The solution was then cooled and the volume was made up to 20 mL with double distilled water. The solution was filtered through Whatman No. 1 filter paper. The cations were estimated from the aliquots of filtrate. Sodium and potassium ions were estimated by using Flame photometer (Elico CL 360) while calcium and magnesium ions were estimated by using atomic absorption spectrophotometer (GBC 932 Plus).

2.5 Statistical analysis

Analysis of variance was used to test the significance of differences between the mean values of six independent observations. Tukey's multiple comparison test (Tukey, 1956) was used to find significance of differences in mean levels of cation of the races, treatments and day wise changes. Differences were considered significant at $P < 0.05$.

3 RESULTS

3.1 Haemolymph Na^+ concentration

The larvae of NB_4D_2 showed higher mean levels of Na^+ (12.3 meq/L) than CSR_2 (11.1 meq/L) and PM (8.54 meq/L) (Table 1). Mean haemolymph Na^+ levels were higher when the larvae were fed on V-1 (12.9 meq/L) than S-36 (10.1 meq/L) and M-5 (8.80 meq/L). Na^+ levels in larval haemolymph were relatively low at 4th moult and showed further fall in post moult (0 d) larvae (Fig. 1). But, a significant increase in Na^+ levels was observed during active feeding period in 5th instar larvae. A significant drop in the Na^+ levels was observed on 7th day when the larvae stop feeding.

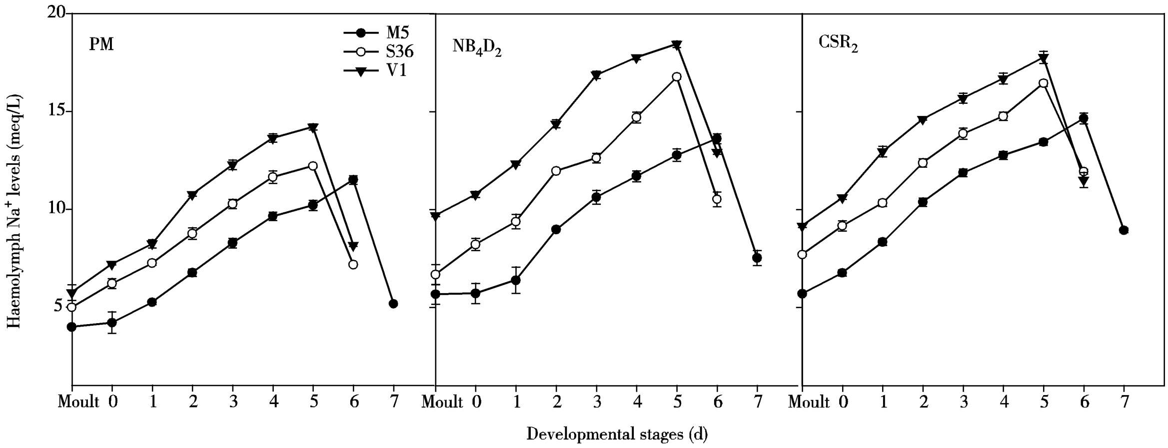

Fig. 1 Sodium levels of haemolymph on different days of larval development of the silkworm races reared on M-5 (control), S-36 and V-1 mulberry varieties

Each value is the mean ± *SD* of 6 separate replications. The same below.

3.2 Haemolymph K^+ concentration

K^+ levels in the haemolymph were significantly higher than Na^+ levels. NB_4D_2 showed higher K^+ (74.3 meq/L) than CSR_2 (67.8 meq/L) and PM

(50.6 meq/L) (Table 1). Haemolymph K^+ levels were higher in larvae feeding on V-1 (68.3 meq/L) than those feeding on S-36 (60.8 meq/L) and M-5 (56.6 meq/L). K^+ levels in larval haemolymph were relatively low during 4th moult and showed further drop in post moult larvae (Fig. 2). K^+ levels increased significantly during active feeding period from 1st day to 6th day. A significant drop in the K^+ levels was observed on 7th day when the larvae stop feeding.

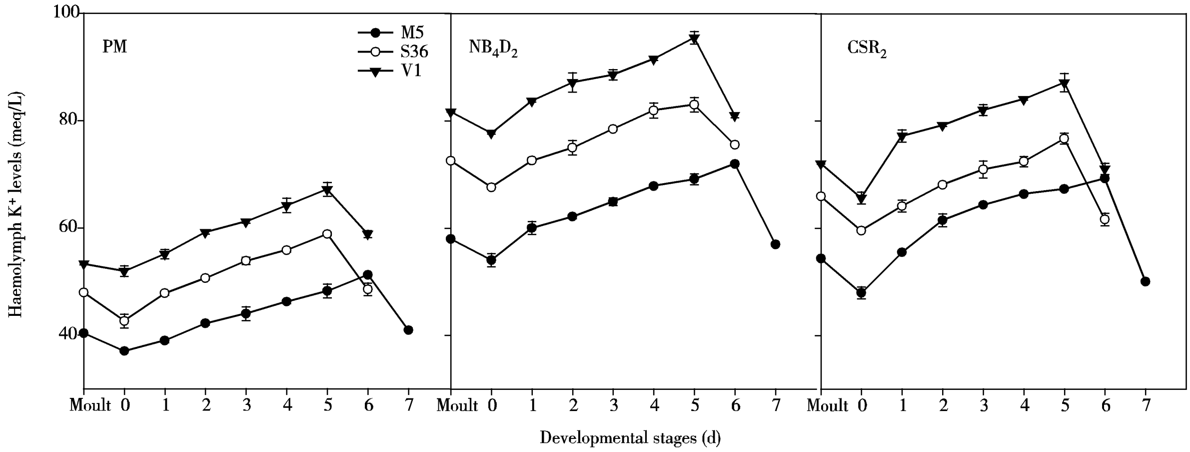

Fig. 2 Potassium levels of haemolymph on different days of larval development of the silkworm races reared on M-5 (control), S-36 and V-1 mulberry varieties

3.3 Haemolymph Ca^{2+} concentration

The larvae of NB_4D_2 showed a relatively higher haemolymph Ca^{2+} level (19.7 mg/mL) than CSR_2 (18.9 meq/L) and PM (13.6 meq/L) (Table 1). Higher haemolymph Ca^{2+} levels were observed in larvae feeding on V-1 (25.1 meq/L) than those feeding on S-36 (21.1 meq/L) or M-5 (15.6 meq/L). Haemolymph Ca^{2+} showed a significant drop in post moult larvae and an increase during active feeding period from 1st day to 6th day (Fig. 3). A significant drop in the Ca^{2+} levels was observed on 7th day when the larvae stop feeding.

3.4 Haemolymph Mg^{2+} concentration

Haemolymph Mg^{2+} level of 5th instar larvae was relatively lower than K^+. NB_4D_2 larvae showed a significantly higher Mg^{2+} level (61.8 meq/L) than CSR_2 (57.8 meq/L) and PM (40.2 meq/L) larvae (Table 1). Higher Mg^{2+} levels were observed when the larvae were fed on V-1 (56.3 meq/L) than on S-36 (51.3 meq/L) or M-5 (45.0 meq/L). The Mg^{2+} level showed a significant drop in post moult larvae (0 d) and a linear increase during active feeding period from 1st day to 6th day (Fig. 4). A significant drop in the Mg^{2+} levels was observed on 7th day when the larvae stop feeding.

Table 1 Summary of ANOVA showing the interaction of mulberry varieties on haemolymph cation levels in different races of *Bombyx mori*

Treatments	Na^+ (meq/L)	K^+ (meq/L)	Ca^{2+} (meq/L)	Mg^{2+} (meq/L)
Mulberry variety				
M-5	8.80 ± 3.29 x	56.6 ± 11.4 x	15.6 ± 3.97 x	45.0 ± 10.8 x
S-36	10.1 ± 2.31 y	60.8 ± 10.5 y	21.1 ± 3.71 y	51.3 ± 9.90 y
V-1	12.9 ± 3.23 z	68.3 ± 12.8 x	25.1 ± 3.71 z	56.3 ± 9.90 z
F-test	**	**	**	**
Race				
PM	8.54 ± 3.08 a	50.6 ± 5.97 a	13.6 ± 4.74 a	40.2 ± 8.61 a
NB_4D_2	12.3 ± 3.14 c	74.3 ± 7.23 c	19.7 ± 4.42 c	61.8 ± 8.73 c
CSR_2	11.1 ± 3.27 b	67.8 ± 8.51 b	18.9 ± 4.53 b	57.8 ± 8.25 b
F-test	**	**	**	**

** Significant at 1% ($P < 0.01$); means with different letters are significantly different from each other (as indicated by Turkey's HSD)

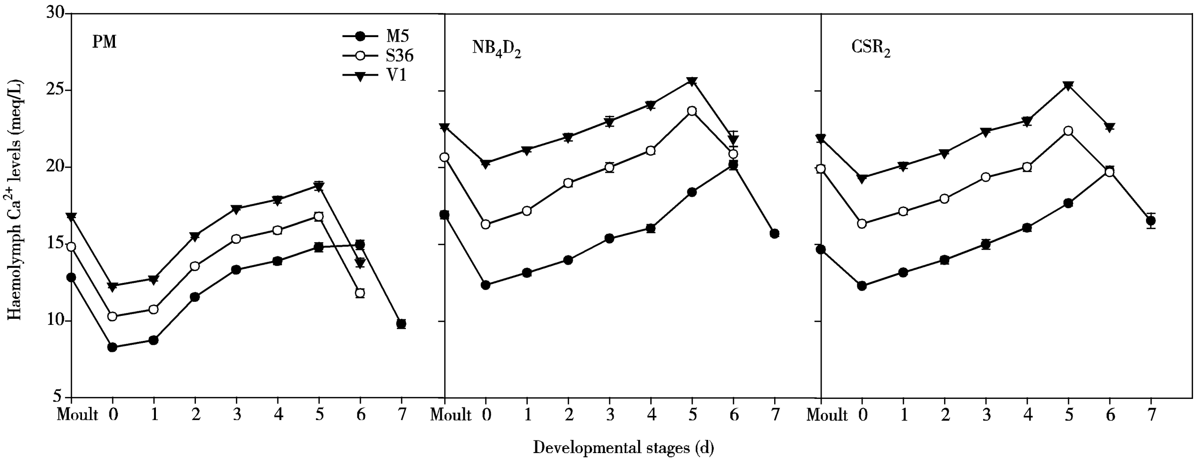

Fig. 3 Calcium levels of haemolymph on different days of larval development of the silkworm races reared on M-5 (control), S-36 and V-1 mulberry varieties

3.5 Cation levels of mulberry leaf and silkworm excreta

The levels of Mg^{2+} in the mulberry leaf were relatively high followed by K^+, Ca^{2+} and Na^+ (Table 2). Among the mulberry varieties, the cation levels were found to be significantly higher in V-1 followed by S-36 and M-5. The levels of cations in the excreta were in the order of $K^+ > Mg^{2+} > Ca^{2+} > Na^+$ (Fig. 5). The rates of excretion of cations were significantly higher in PM than CSR_2 and NB_4D_2 (Table 3). Lower cation levels were observed in larvae feeding on M-5 than those feeding on S-36 and V-1 (Table 3). The cation levels in excreta were relatively higher during 4th moult, post moult (0 d) and spinning larvae (7th day) (Fig. 5). In the feeding larvae, lower cation levels were observed in the excreta from 1st day to 6th day. K^+ was excreted more than the other three cations.

Table 2 Analysis of variance for cation levels of leaves of selected mulberry varieties

Treatment	Na^+ (meq/kg dry weight)	K^+ (meq/kg dry weight)	Ca^{2+} (meq/kg dry weight)	Mg^{2+} (meq/kg dry weight)
Mulberry variety				
M-5	120 ± 0.01 a	332 ± 0.02 a	138 ± 0.02 a	516 ± 0.01 a
S-36	124 ± 0.01 b	338 ± 0.03 b	146 ± 0.02 a	540 ± 0.02 b
V-1	131 ± 0.02 c	443 ± 0.05 c	153 ± 0.03 b	565 ± 0.01 c
F-test	**	**	*	**

** Significant at 1% ($P < 0.01$); * Significant at 5% ($P < 0.05$); means with different letters are significantly different from each other (as indicated by Turkey's HSD), each value is the mean ± *SD* of three separate observations. The same for Table 3.

Table 3 Analysis of variance for cation levels in silkworm excreta in different races of *Bombyx mori*

Treatment	Na^+ (meq/kg dry weight)	K^+ (meq/kg dry weight)	Ca^{2+} (meq/kg dry weight)	Mg^{2+} (meq/kg dry weight)
Mulberry variety				
M-5	14.9 ± 3.29 x	113 ± 11.4 x	20.5 ± 3.97 x	58.9 ± 10.8 x
S-36	17.8 ± 2.31 y	123 ± 10.5 y	26.3 ± 3.71 y	71.2 ± 9.90 y
V-1	19.9 ± 3.23 z	133 ± 12.8 x	30.3 ± 3.71 z	81.1 ± 9.90 z
F-test	**	**	**	**
Race				
PM	18.2 ± 1.08 c	127 ± 5.97 c	28.7 ± 4.74 c	86.5 ± 8.61 c
NB_4D_2	17.2 ± 3.14 b	124 ± 7.23 b	25.2 ± 4.42 b	62.5 ± 8.73 b
CSR_2	16.1 ± 3.27 a	121 ± 8.51 a	22.4 ± 4.53 a	60.7 ± 8.25 a
F-test	**	**	**	**

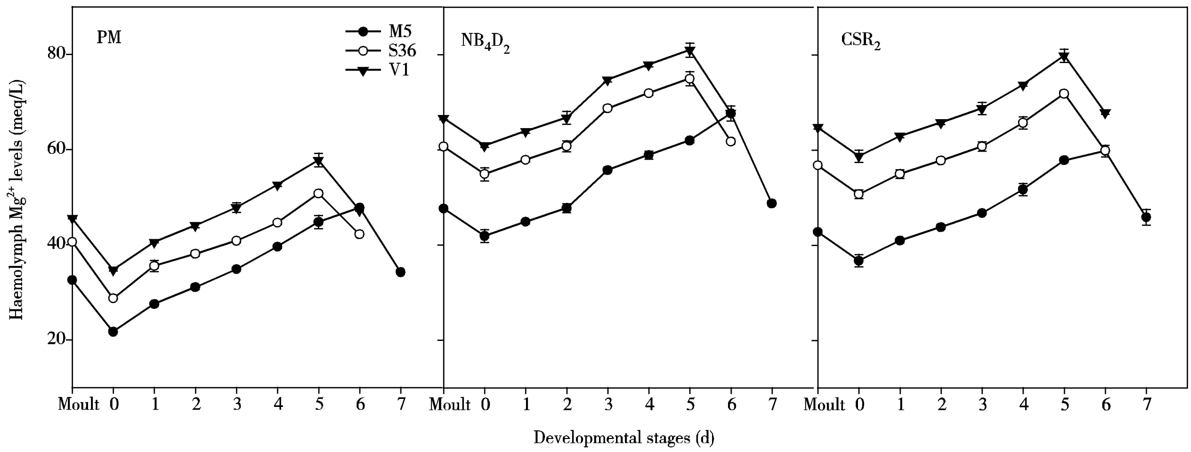

Fig. 4 Magnesium levels of haemolymph on different days of larval development of the silkworm races reared on M-5 (control), S-36 and V-1 mulberry varieties

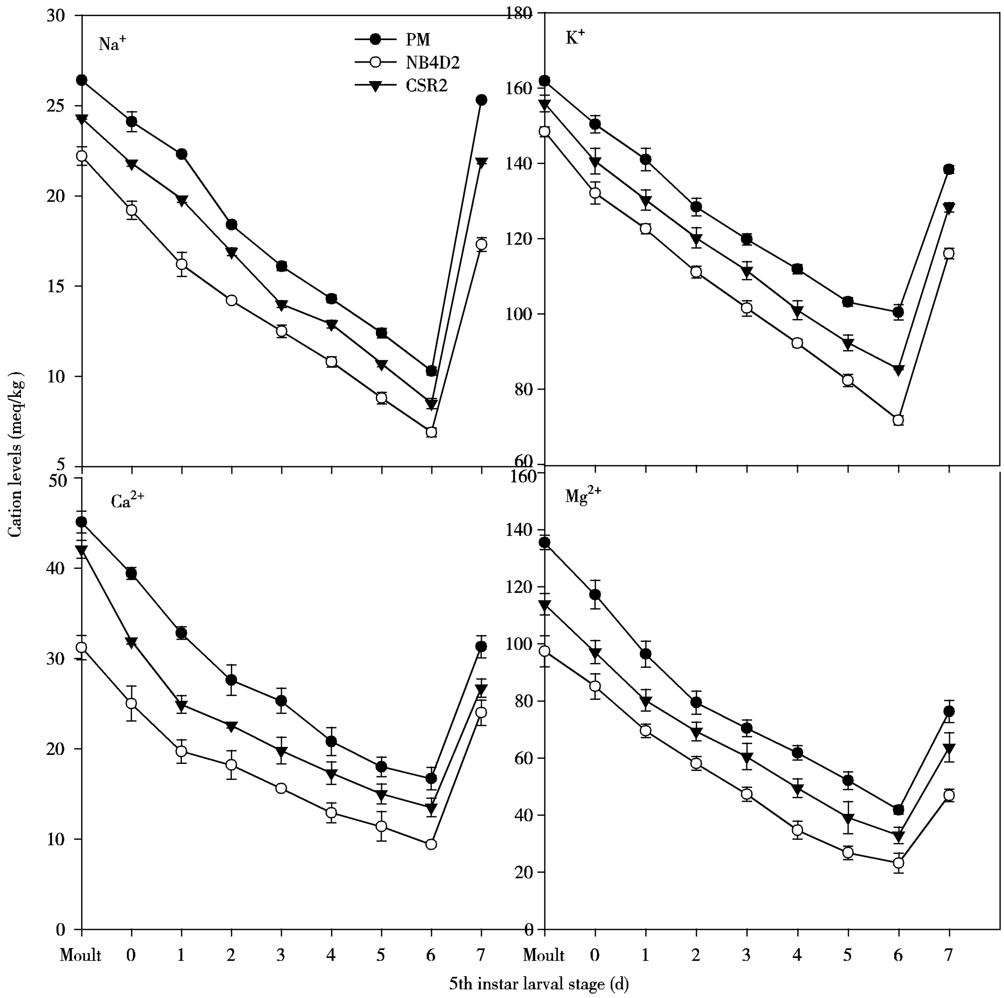

Fig. 5 Cation levels in the silkworm excreta in different races of *Bombyx mori* during the 5th instar of larval development

4 DISCUSSION

4.1 Haemolmph cation levels

Haemolymph cation composition is dietetic; the relative levels of cations in the diet are reflected in the composition of the haemolymph. K^+ and Mg^{2+} levels were high in the leaves (Table 2) and also in the haemolymph (Figs. 2 and 4). Haemolymph cation levels increased significantly throughout the feeding period from 1st to 6th day in all the three races. Diet, temperature and disease influence haemolymph cationic composition of an insect (Mullins, 1985). The silkworm haemolymph plasma is characterized by low

Na^+ and very high K^+ and Mg^{2+} concentrations and moderately high Ca^{2+} like in the mulberry leaf. Weevers (1965) reported similar haemolymph ionic composition in the Tasar silkworm, *Anthaeraea mylitta*.

4.2 Changes in the cation levels during development

The haemolymph cation levels were relatively high during moult but less in post moult larvae. An increase in haemolymph cation concentration was observed during 4th moult in the larvae of the giant silk moth, *Hyalophora cecropia* (Harvey *et al.*, 1975). Ziegler *et al.* (2000) observed that a sudden increase in the haemolymph volume between late premoult and intramoult served to expand the cuticle during moult in the isopod, *Ligia pallasii*. The drop in the cation levels from moult to post moult stage can be attributed to swift changes occurring in the fluid levels of haemolymph during moult cycle.

4.3 Changes in cation levels take place by controlled excretion

Cation levels during feeding stage are determined by relative rates of their absorption and excretion and on the cationic composition of the leaf and larval requirement. Mg^{2+} level is higher than K^+ in mulberry leaf but in feeding larvae K^+ was excreted in significantly higher quantities than Mg^{2+} to prevent Na^+/K^+ ratio dropping low when high haemolymph Mg^{2+} levels affect neural function. Also, lower excretory rates of Na^+ lead to further improvement of Na^+/K^+ ratio. Harvey and Zerahn (1971) proposed a Na^+ pump in Cecropia midgut which was unaffected by high K^+. A negative correlation existed between the levels of cations in haemolymph and excreta. The haemolymph cation levels are held by controlled excretion of the cations (Figs. 1–5). Na^+ was the least while K^+ was the highest in excreta of feeding larva. Elimination of cations was least during feeding stage suggesting their more complete absorption during growth phase.

4.4 Spinning larvae showed an acute drop in haemolymph cations

The haemolymph cation levels in spinning stage drop by increased rates of their excretion (Figs. 1–5). The spinning larvae shrink in size and store large quantities of amorphous silk in the lumen of the silk gland. The immediate and proximate source of water for silk biosynthesis is haemolymph and the consequent volume reduction of haemolymph requires elimination of cations to maintain the osmotic pressure (Jungreis *et al.*, 1973).

4.5 Regulation of haemolymph monovalent cations

Mulberry variety also greatly influenced Na^+ of larval haemolymph. Higher Na^+ of S-36 and V-1 coupled with higher leaf moisture content facilitates more efficient absorption of Na^+ across the midgut epithelium. Haemolymph Na^+ was relatively higher in NB_4D_2 and CSR_2 than in multivoltine PM due to higher levels of leaf ingestion in the bivoltine races. Among the three factors, *viz.* ontogeny, mulberry variety and silkworm race that influence haemolymph K^+, the relative effect of race was the highest (Tables 1–3). K^+ levels were held constant throughout larval development and the influence of mulberry variety was also not significant. Efficient regulation of haemolymph K^+ by active transport has been suggested (Harvey and Nedergaard, 1964). Very high haemolymph K^+ levels also reduce the efficiency of Na^+ pump (Harvey and Zerahn, 1971). But K^+ levels of bivoltine races were about 30% higher than the multivoltine PM. Higher osmolar concentration of haemolymph of bivoltine races provides a favorable passive absorption gradient of digested nutrients in the lumen to haemolymph across midgut epithelium.

4.6 Haemolymph Ca^{2+} levels are more stable

Haemolymph Ca^{2+} levels do not change significantly during 5th instar larval development though they are slightly higher in bivoltine races and in silkworms fed with V-1 and S-36 leaf. Higher Ca^{2+} levels could be associated with higher productivity since Ca^{2+} is secreted as a constituent of silk. During moulting, a significant decrease in haemolymph Ca^{2+} was observed. The role of Ca^{2+} in moulting cycle in insects has not been thoroughly investigated as in other invertebrates like crustaceans having a calcareous shell. Ziegler *et al.* (2000) observed significant increase in the haemolymph Ca^{2+} from the inter-moult to intra-moult and post-moult due to resorption of Ca^{2+} from the cuticle and external $CaCO_3$ deposits.

4.7 Haemolymph Mg^{2+} levels depend on dietary intake

Higher haemolymph Mg^{2+} concentration was observed during moult in all the three races. A significant drop in the haemolymph Mg^{2+} concentration from moult to post-moult (0 d) suggests dietary supply cut off. A similar decrease in haemolymph Mg^{2+} levels in prepupal blood was reported (Chapman, 1998). A significant increase in haemolymph Mg^{2+} during feeding stage, and its higher levels in bivoltine races and in larvae fed with S-36 and V-1 also signify dietary source. The rates of leaf ingestion in bivoltine races are significantly higher and elite mulberry varieties have more cholorophyll content than M-5 (Mahadeva, 2006). The relatively higher Mg^{2+} levels don't cause any anesthetic effect because of antagonistic action of

high haemolymph Ca^{2+} levels. Also, the divalent cations may exist in partially bound form associated with hemolymph proteins or organic acids .

4.8 General conclusions

The haemolymph cations of the silkworm, *Bombyx mori* are derived from the mulberry leaf. Na^{+}/K^{+} and Mg^{2+}/Ca^{2+} ratios were held in limits by greater excretion of K^{+} and Mg^{2+} in view of their adverse effects on neural function at high concentration. Though the cationic composition is influenced by race, ontogeny and quality of the leaf, the disparate leaf ingestion levels in different silkworm races leave a stronger impact of the race on haemolymph cationic composition. The cation levels were held by controlled excretion as significant volume changes occur during feeding, moult, post moult and spinning larval stages.

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桑叶营养对家蚕血淋巴阳离子水平的调节作用

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摘要: 调查了取食不同品种桑树(M-5, S-36 和 V-1)叶片的家蚕(多化性品种 Pure Mysore、二化性品种 NB_4D_2 和 CSR_2)5 龄幼虫血淋巴中阳离子的变化。结果表明: 家蚕幼虫血淋巴中 Na^{+}浓度低, K^{+}和 Mg^{2+}浓度很高, Ca^{2+}浓度较高。在幼虫活跃取食期间, 血淋巴中阳离子水平显著提高。血淋巴中阳离子水平与排泄物的量呈负相关。血淋巴中阳离子水平受阳离子外泌的控制。与非取食阶段相比, 取食阶段的阳离子清除速率低。家蚕二化性品种的血淋巴阳离子水平比多化性品种 PM 高出 30%。结果说明家蚕品种对家蚕血淋巴阳离子变化的影响显著大于桑树品种或家蚕个体发育的影响。

关键词: 家蚕;桑叶;营养;血淋巴;阳离子水平

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